Expression of the Murine Plasma Cell Nucleotide Pyrophosphohydrolase PC-1 Is Shared by Human Liver, Bone, and Cartilage Cells

Regulation of PC-1 Expression in Osteosarcoma Cells by Transforming Growth Factor-β

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Abstract

A bone and cartilage enzyme with both 5′-nucleotide phosphodiesterase I and nucleotide pyrophosphohydrolase (NTPPPH) activity modulates physiologic mineralization and pathologic chondrocalcinosis by generating inorganic pyrophosphate. We hypothesized that, as for alkaline phosphatase, expression of an NTPPPH gene can be shared by cells from bone, cartilage, and liver and by certain leukocytes.

Recently, we demonstrated the hepatocyte and murine plasma cell membrane glycoprotein PC-1 to have both 5′-nucleotide phosphodiesterase I and NTPPPH activity. We detected polypeptides cross-reactive with PC-1 in human U20S osteosarcoma cells, articular chondrocytes, homogenized human knee cartilages, human knee synovial fluids, hepatoma cells, and murine plasmacytoma cells. Constitutive low abundance PC-1 mRNA expression was detected in U20S cells and chondrocytes by a nested RNA–PCR assay and by Northern blotting. TGFβ is known to substantially increase NTPPPH activity in primary osteoblast cultures. We demonstrated that TGFβ1 increased NTPPPH activity and the level of PC-1 mRNA and immunoprecipitable [^{35}S]-methionine-labeled PC-1 polypeptides in U20S cells.

The identification of PC-1 as an NTPPPH expressed in cells derived from bone and cartilage may prove useful in furthering the understanding of the role of NTPPPH in physiologic and pathologic mineralization. (*J. Clin. Invest.* 1994. 94:560–567.) Key words: PC-1 • nucleoside triphosphate pyrophosphohydrolase • bone mineralization • chondrocalcinosis • transforming growth factor-β

Introduction

Membrane-bound enzyme activity that hydrolyzes the nucleoside monophosphate-pyrophosphate (phosphodiester I) bond in nucleotides (termed nucleotide pyrophosphohydrolase, abbreviated NTPPPH)[1] is expressed by osteoblasts, chondrocytes, and many other tissues (e.g., placenta, erythrocytes, fibroblasts, and hepatocytes [1–4]). NTPPPH participates in a cascade system of enzymes that hydrolyze nucleotides and nucleic acids to nucleosides (5). This enzyme chain permits the salvage of nucleotides from extracellular fluids such as bile (5) and the uptake of nucleosides by cells that do not rely on synthesis of purines by the de novo pathway (6, 7). Furthermore, scavenging of extracellular ATP released by injured and dividing cells may be necessary to prevent tissue injury triggered by ATP-induced cellular activation (8).

In bone and cartilage, NTPPPH-catalyzed release of inorganic pyrophosphate (PPi) from nucleoside triphosphates provides a substrate for alkaline phosphatase–catalyzed generation of Pi and the crystallization of basic calcium phosphates (9–13). The role of NTPPPH activity in physiologic endochondral mineralization is reinforced by the presence of NTPPPH in growth plate cartilage matrix vesicles (11–15). NTPPPH activity is also linked to pathologic mineralization of adult hyaline articular cartilage, i.e., deposition of crystals of hydroxyapatite and other basic calcium phosphates (16), and of calcium pyrophosphate dihydrate (CPPD) (in CPPD crystal deposition disease, or chondrocalcinosis) (17–20). Specifically, adult cartilage, like osteoblasts and growth plate cartilage, contains matrix vesicles (21, 22), and the addition of an exogenous NTPPPH substrate (ATP) (23) to purified human adult cartilage matrix vesicles and to cartilage promotes the generation of CPPD-like crystals at physiologic pH (22, 24).

Bone, cartilage, and other tissue NTPPPH enzymes (1–4, 9, 10, 25–27) share the capacity to recognize and hydrolyze the phosphodiester I bond in a variety of nucleotides and synthetic nucleoside substrates (e.g., nucleotide sugars, 3′-phosphoadenosine 5′-phosphosulfate), are "ectoenzymes" (i.e., functionally active on the extracellular face of the plasma membrane), and have an alkaline pH optimum.

The genes responsible for human bone and cartilage enzymes with NTPPPH activity are unknown. Importantly, one gene for another ectoenzyme, L/B/K alkaline phosphatase, can be expressed in different forms by hepatocytes, osteoblasts, and chondrocytes and certain granulocytic cells (28). Thus, we hypothesized that, like certain other ectoenzymes, at least one NTPPPH species in osteoblasts and cartilage may be expressed by bone and cartilage cells, liver cells, and also serve as a

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1. *Abbreviations used in this paper:* CPPD, calcium pyrophosphate dihydrate; NTPPPH, nucleotide pyrophosphohydrolase; PC-1, plasma cell membrane glycoprotein-1; PPi, inorganic pyrophosphate.

marker for the state of differentiation of certain leukocytes (28, 29). In this regard, we noted that the 5′-nucleotide phosphodiesterase I, PC-1, which was first recognized as a differentiation marker for murine antibody-secreting cells of B lymphocyte origin (30–34), is also expressed in bovine hepatocytes (35) and that antibodies to PC-1 reacted with an undefined species by immunostaining in murine hyaline cartilage (36). Cloning and functional characterization of PC-1 has been carried out recently by us (30, 32–34, 37). We have demonstrated that PC-1 is an ectoenzyme that acts intracellularly and extracellularly and functions as a PPi-generating NTPPPH in both the intracellular and extracellular milieus (38). Furthermore, PC-1 can be expressed in at least one soluble extracellular form (39). Therefore, this study tested the hypothesis that cells from human bone and cartilage may share the capacity of hepatocyes and murine plasma cells to express the NTPPPH PC-1.

Methods

Tissue sources and culture conditions. A human osteosarcoma cell line with known osteoblast-like characteristics (U2OS) (40) was obtained from American Type Culture Collection (Rockville, MD) and maintained in McCoy's 5A medium supplemented with 15% FCS, 1% penicillin–streptomycin, and 2 mM glutamine. A human hepatoma (HepG2) cell line also was obtained from the American Type Culture Collection repository. The murine plasmacytoma cell line, NS-1, which spontaneously expresses PC-1, was cultured as described previously by us (34).

Cartilage and chondrocytes. The cartilages described in these experiments were obtained from the femoral condyles and tibial plateaus of osteoarthritis patients at the time of total knee joint replacement. All chondrocytes were first isolated within the laboratory of Dr. Martin Lotz (UCSD) by methods outlined previously in detail (41, 42). After removal of blood and tissue debris, and to avoid chondrocyte culture contamination by other articular tissues, the articular surface was scraped gently with a scalpel from which any tissues stuck from the synovial surface or from synovial fluid were removed. Second, cartilage was collected only from noncalcified areas and from soft cartilage. Fibrillated cartilage was not used at all. Cartilage was minced with a scalpel and treated with trypsin (10% vol/vol) for 15 min in a 37°C waterbath. The samples were transferred to DME containing 5% FBS, penicillin-streptomycin-fungizone, and 2 mg/ml clostridial collagenase type IV (Sigma Immunochemicals, St. Louis, MO) and digested for 3 h on a gyratory shaker until the tissue fragments were dissolved.

Subcultured cells (passages 1–5) were trypsinized from T175 flasks and plated in 96-well plates at 5,000–20,000 cells/well in DME 1% FBS. The media were removed, the wells were washed twice with serum-free DME, and culture was continued in DME supplemented with L-glutamine and antibiotics.

Synovial fluids. Human knee synovial fluids were obtained by informed consent during diagnostic or therapeutic procedures in noninflammatory conditions (osteoarthritis, internal derangement). All were nonhemorrhagic, noninflammatory by transparency and viscosity, and free of crystals by polarized light microscopy. All fluids were treated with EDTA (5 mM), PMSF (100 μM) and aprotinin (0.01%), and testicular hyaluronidase (100 μg/ml) and centrifuged (6,900 g for 30 min at 4°C) to remove cells and particulates.

5′-Nucleotide phosphodiesterase I assay. The assay was performed using as substrate 1 mM p-nitrophenyl-thymidine 5′-monophosphate (PNTM) (Sigma Immunochemicals) in 50 mM Hepes-buffered DME containing 1.6 mM $MgCl_2$, pH 8.0, in a volume of 0.5 ml to which 0.05 ml of sample was added for 1 h (10, 27). The assay was halted by addition of 4 vol of 100 mM NaOH, and absorbance at OD 405 was determined. Specific enzyme activity was measured against a standard curve of p-nitrophenol and expressed in Units (1 U was equivalent to 1 μmol of substrate hydrolyzed per h).

Anti-PC-1 antibodies. We used two previously described rabbit polyclonal antisera to recombinant murine PC-1 clones (3-1 and 5) and an antiserum to chromatographically purified, denatured mouse PC-1 (ADPC-1) (34). In addition, two antisera to recombinant human PC-1 (R1040 and R1042) were generated by immunizing rabbits with a synthetic peptide consisting of the COOH-terminal 15 amino acids of human PC-1, coupled to bovine serum albumin using glutaraldehyde, and emulsified in complete Freund's adjuvant. A 50% saturated ammonium sulfate cut was isolated from each anti-human PC-1 antiserum and from control nonimmune rabbit serum and was washed twice by passive adsorption to an equal volume of human erythrocytes before being used.

Western blot detection of PC-1. Lysates of washed cells were generated by treatment for 10 min in 1% Triton X-100. SDS-PAGE and Western blotting were performed largely by previously described methods (43). Specifically, electrophoretic transfer of proteins previously separated in an 8% SDS-polyacrylamide gel to polyvinylidene difluoride was performed at 450 mA for 90 min at 4°C. Blots previously saturated with blocking buffer (0.25% powdered nonfat milk, 0.12% Tween 20, in SSC) were incubated overnight with anti-PC-1 primary antibody (at 1:2,000 in blocking buffer), washed twice, and incubated with alkaline phosphatase–conjugated goat anti-rabbit IgG (in blocking buffer at 1:2,000) for 1 h. After two more washes, the blot was washed in assay buffer (100 mM diethanolamine, 1 mM $MgCl_2$, in ddH_2O) and then in chemiluminescent enhancing solution (5% reagent A, FLASH Western Blot System; Stratagene, La Jolla, CA). The blot was then incubated with the kit's chemiluminescent reagent in assay buffer for 10 min and then exposed to x-ray film for 10–30 min.

Immunoprecipitation of PC-1 activity. Cells were spun down and washed in 150 mM NaCl, 10 mM Tris (pH 7.6) and resuspended for 24 h at 4°C in the same buffer to which 0.1% Triton X-100 was added. Cell lysates (40 μg protein in a total volume of 0.02 ml) were incubated with 0.01 ml of nonimmune rabbit serum or the same volume of anti-PC-1 antisera (a combination of 0.002 ml each of R1040, R1042, 3-1, 5, and ADPC-1) overnight at 4°C. Staph protein A–Sepharose, 0.05 ml of a 50% suspension, was then added for 2 h at 4°C with constant agitation, then this suspension was sedimented in a Stratagene picofuge (model #400550) for 30 s. The beads were then washed twice in 1.0 ml of the Tris/NaCl buffer, as above. To the pelleted beads, or the supernatant (0.05 ml), was added 0.5 ml of buffer containing 1 mM PNTM, 15 mM Hepes, and phenol-free DME, pH 7.4, for 1 h.

For [^{35}S]methionine labeling, confluent U2OS cells ($\sim 0.5 \times 10^6$ cells in 10 ml) were labeled with 800 μCi [^{35}S]methionine (NEN, Boston, MA) for 6 h in methionine-free DME (GIBCO BRL, Baltimore, MD) and chased for 24 h in complete DME medium in the presence or absence of recombinant TGFβ (1 ng/ml) for 24 h. Washed, harvested monlayers were lysed in Triton X-100 (1%) for 1 h on ice. The cell lysates (0.5 ml) were precleared with protein G–Sepharose beads (0.05 ml of a 10% solution) (Zymed Laboratories, Inc., South San Francisco, CA) by a 2-h incubation at 4°C, followed by centrifugation at 5,000 g for 30 s. Antibodies (0.01 ml total as above) were added for 18 h. Proportionate concentrations of nonimmune sera were used as controls. Polypeptides were precipitated after adding 0.1 ml of a 10% solution for 1.5 h at 4°C. Pellets were washed three times in 5 vol PBS, boiled, and then analyzed by SDS-PAGE. After staining with Coomassie blue, the gels were treated with Amplify (Amersham Corp., Arlington Heights, IL) for 1 h, dried, and exposed to Kodak XAR film at −70°C for 4 h.

Detection of PC-1 mRNA. A full-length human PC-1 cDNA hybridization probe in pBluescript SK- was constructed by ligation of two partial cDNA clones from placental libraries (33). Restriction enzyme mapping and sequencing of the extreme 5′ end of the human PC-1 cDNA construct confirmed identity with human PC-1.

For Northern blotting, mRNA was purified using the Pharmacia LKB Biotechnology Inc. (Piscataway, NJ) "quick mRNA prep kit" according to the manufacturer's instructions. Where indicated, total RNA was isolated by a previously described method using guanidinum isothiocyanate (41). Samples were blotted onto nitrocellulose from a 1.2% agararose/2.2 M formaldehyde gel by capillary transfer. Hybrid-

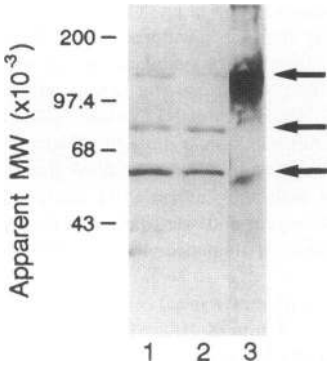

Figure 1. PC-1 polypeptide expression in human hepatoma and osteosarcoma cells and murine plasmacytoma cells. Cell lysates were studied by SDS-PAGE and Western blotting, using the anti–PC-1 antiserum, 3-1, as described in Methods. Lane *1*, U2OS osteosarcoma cells, 15 μg; lane *2*, HepG2 hepatoma cells, 15 μg; lane *3*, murine plasmacytoma (NS-1 cells, 25 μg); lane *3* is from a separate gel, for which molecular mass standards are not provided. Molecular mass markers are as indicated on the left. Large arrows identify species of ~ 130, 86, and 60 kD.

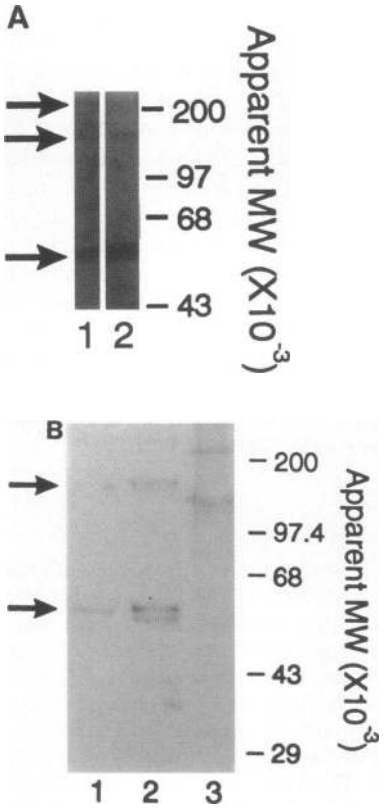

Figure 2. PC-1 polypeptides are expressed in monolayer-cultured human articular chondrocytes. SDS-PAGE and Western blotting were performed as described above, using anti–PC-1 antiserum 3-1. Arrows indicate polypeptides of ~ 260, 130, and 60 kD. (*A*) Lane *1*, second passage articular cartilage chondrocytes obtained from surgical specimen of an 80-yr-old man with osteoarthritis of the hip (25 μg); lane *2*, osteosarcoma cell lysate (25 μg). (*B*) Lane *1*, second passage articular chondrocytes from a 72-yr-old man with osteoarthritis of the knee (30 μg); lane *2*, hepatoma cell lysate (30 μg); lane *3*, plasmacytoma cell lysate (15 μg).

ization was to random-primed ^{32}P-labeled recombinant human PC-1 (5×10^6 cpm/ml) at 65°C for 16 h. After hybridization, blots were washed twice at 22°C for 15 min in 5 × SSC, 0.1% SDS, and then twice at 65°C in 0.5 × SSC, 0.1% SDS for 15 min.

For RNA–PCR, we selected primers that spanned more than one intron (based on knowledge of human PC-1 5′ intron–exon structure [39]). We prepared a double-stranded cDNA template from 10^7 U2OS cells by isolating 5 μg of total RNA and reverse-transcribing it using random primers and the Invitrogen (San Diego, CA) RT-cDNA PCR kit. One-twentieth of the cDNA was used as a template for two rounds of PCR reaction, using the 5′ and 3′ primers for PC-1 (300 ng each) as indicated below, and 250 μM dNTPs, and 2.5 U of AmpliTaq (Perkin-Elmer/Cetus, Norwalk, CT) in 1.5 mM magnesium containing–buffer (conditions: an initial 4-min run at 94°C before starting, then 94°C at 2 min, 55°C at 2 min for annealing, and 72°C at 3 min for extension. The final extension, at the end of 40 cycles, was for 8 min at 72°C).

PC-1 RNA–PCR primers were as follows: first round 5′ primer: CCA ACA CCT ATA AAG TAC TCT CGC TGG; 3′ primer ATC CGG TGA CAA TGC TGT AGT GAT TGG; second round of PCR: 5′ primer CCA AGG TAC CTA AAG TAC TCT CGC TGG (starting at base 232 of the coding region of PC-1); 3′ primer CTG TGG ATC CTT AAT GCT CTC ACA TGG (starting at base 603 of the coding region).

As a "housekeeping" gene control, we performed a single round of RNA–PCR on the same cDNA templates for the liver ribosomal protein, L30 (44), which we obtained from Dr. Jerrold Olefsky (UCSD). The L30 sense primer was 5′-GAAAGTACGTGCTGGGGTACAAACAGACTC, and the L30 antisense primer was 5′-ATCGGAATCTGGGTCAATGATAGCCAG.

Results

Osteosarcoma cells and human articular chondrocytes express polypeptides cross-reactive with PC-1 in vitro. To test the possibility that PC-1 is expressed by cells of human bone and cartilage, we first compared cell lysates from human chondrocytes, osteosarcoma cells, hepatoma cells, and murine plasmacytoma cells by SDS-PAGE and Western blotting. PC-1 is expressed as a membrane homodimer of 115–130-kD monomers and also as smaller soluble peptides that may be generated via proteolysis (39). PC-1 polypeptides in murine plasmacytoma cells were confirmed (34, 37) to be expressed as a predominant full-length species with a molecular mass of ~ 115–130 kD and as a minor species of ~ 230, 90, and 60 kD (Fig. 1, lane *3*). Osteoblast-like U2OS osteosarcoma cells (40) and a human transformed hepatocyte cell line (HepG2) expressed PC-1 cross-reactive polypeptides that included low abundance species of ~ 130 and 86 kD and a higher abundance species of 60 kD (Fig. 1, lanes *1* and *2*).

In human articular chondrocytes, antimurine recombinant PC-1 antibodies also detected low abundance polypeptide species with estimated molecular masses of 260, 130, and 60 kD (Fig. 2). In all cells tested, the PC-1 cross-reactive polypeptides of 60 kD became more abundant, and the higher molecular mass species became less abundant as samples were stored for longer periods of time (data not shown), suggesting that the 60-kD species might arise from degradation of parent PC-1 molecules.

Thus, the capacity for PC-1 expression appeared to be shared by murine plasma cells, human hepatoma cells, and human osteosarcoma cells and chondrocytes. PC-1 also was expressed by whole cartilage in the joint space in vivo. Specifically, knee articular cartilage homogenates from osteoarthritis patients contained polypeptides compatible with full-length PC-1 species (Fig. 3, lanes *1* and *2*). Smaller polypeptides that were cross-reactive with PC-1 were also present in cartilage homogenates (Fig. 3, lanes *1* and *2*). Furthermore, in the course of storing cartilage samples, as with the storage of lysates of cultured cells discussed above, we noted that PC-1 cross-reactive polypeptides of 60 kD became more abundant, and larger polypeptides became less plentiful with time (data not shown).

Because articular cells can export products into the synovial fluid, we ascertained whether PC-1 polypeptides were detectable in human synovial fluids. Eight nonhemorrhagic knee synovial fluids (four uncomplicated osteoarthritis, four internal derangements) all contained PC-1 cross-reactive species, predominantly in the form of a 60-kD polypeptide (Fig. 4). Some PC-1 polypeptides from synovial fluids did not enter the gel well

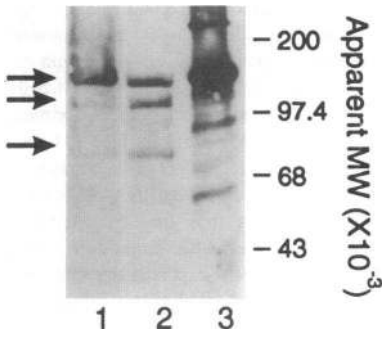

Figure 3. PC-1 cross-reactive polypeptides present in human cartilage in vivo. SDS-PAGE and Western blotting were performed as described above. Lanes *1* and *2*, homogenates of two human osteoarthritic knee cartilages (30 μg of protein). Patients were different from those studied in Fig. 2; lane *3*, plasmacytoma cell lysate as control (30 μg of protein). Arrows indicate polypeptides of ~ 130, 115, and 72 kD.

(Fig. 4); the nature of this cross-reactive material was not investigated.

PC-1 is constitutively a low abundance mRNA in human chondrocytes and osteosarcoma cells. The active site of PC-1 is highly homologous to that of intestinal alkaline phosphodiesterase (45, 46), suggesting that PC-1 could share a conserved enzymatic domain with related but nonidentical enzymes in other tissues. Therefore, to verify that PC-1 was being expressed in chondrocytes, we attempted to detect transcription of PC-1. PC-1 is expressed as a very low abundance mRNA in certain tissues (33, 47). For example, Northern blot hybridization to 10 μg of isolated mRNA was previously required to confirm PC-1 mRNA expression in human fibroblasts under resting conditions (47). Under similar conditions (Fig. 5), using 10 μg of mRNA, we also detected PC-1 mRNA (3.8 kb) on Northern blots from human chondrocytes and osteosarcoma cells. However, because of the low strength of the signal, transcription of

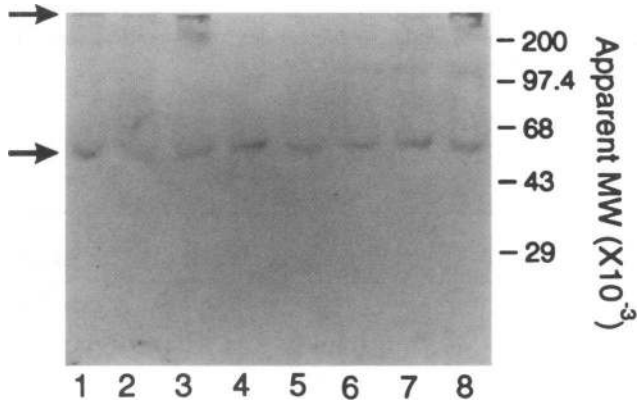

Figure 4. PC-1 cross-reactive polypeptides in human synovial fluids. Grossly noninflammatory synovial fluids (25 μg of protein) from eight separate patients were isolated and treated as described in Methods and studied by SDS-PAGE and Western blotting using antiserum 3-1 as described above. Lanes *1–4* were from patients with osteoarthritis; lanes *5–8* were from patients with internal derangements. Arrows indicate polypeptides of 60 kD in all specimens and high molecular mass material that did not enter the gel in specimens *1–3* and *8*. Faint PC-1 polypeptides of 130 kD were seen in lanes *6–8* but did not reproduce well in photographs.

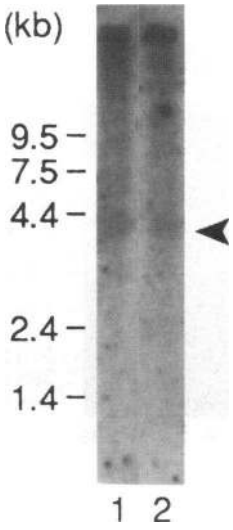

Figure 5. Constitutive low abundance PC-1 mRNA in osteosarcoma cells and cultured articular chondrocytes detected by Northern blotting. 10 μg of mRNA was purified from U2OS cells (lane *1*) and human chondrocytes (lane *2*) using the Pharmacia LKB Biotechnology Inc. "Quick mRNA prep kit" according to the manufacturer's instructions. Samples were blotted onto nitrocellulose from a 1.2% agarose/2.2 M formaldehyde gel by capillary transfer. Hybridization with random-primed ^{32}P-labeled recombinant PC-1 (5×10^6 cpm/ml) was at 65°C for 16 h. After hybridization, blots were washed twice at 22°C for 15 min in 5 × SSC, 0.1% SDS, and then twice at 65°C in 0.5 × SSC, 0.1% SDS for 15 min. The autoradiograph was exposed for 10 d at −70°C. RNA size standards are indicated on the left. *Arrowhead*, 3.8 kb PC-1 mRNA, lanes *1* and *2*.

PC-1 was further verified in chondrocytes and osteosarcoma cells via nested RNA–PCR.

To detect PC-1 by RNA–PCR, we selected primers likely to span at least one intron, based on our knowledge of PC-1 5′ intron–exon structure (39). Using two rounds of PCR, with nested primers, we amplified PC-1 from double-stranded cDNA generated from random-primed, reverse-transcribed total RNA from human monolayer-cultured chondrocytes. Under these conditions, RNA–PCR detected PC-1 as a product of the predicted (370 bp) length in osteosarcoma cells and hepatoma cells (Fig. 6, lanes *1* and *3*) and in murine plasmacytoma (NS-1 cells) (Fig. 7 *B*, lane *4*). RNA–PCR verified PC-1 mRNA expression in human chondrocytes (Fig. 7 *B*, lane *1*). Chondrocytes cultured in the presence of 200 nM dexamethasone (which can enhance PC-1 transcription in certain mouse plasmacytoma cells [34]) and the cartilage growth factor, TGFβ1 (Fig. 7 *B*, lanes *2* and *3*), were verified, in a qualitative manner, to also transcribe PC-1. In parallel, the detection of transcription of a housekeeping gene, the ribosomal protein L30 (44), by a single round of PCR, verified approximately equal loading of cDNA

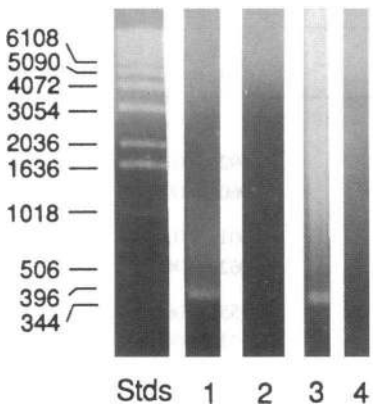

Figure 6. RNA–PCR detects PC-1 transcription in osteosarcoma cells and hepatoma cells. We used two rounds of PCR, with nested primers, to amplify PC-1 from double-stranded cDNA generated from random-primed, reverse-transcribed total RNA (5 μg), as described in Methods. The second round of PCR gave a product of the expected size (381 bp). Standards (RNA ladder) are indicated on the left. Lane *1*, U2OS cells; lane *2*, control for lane *1* (no template); lane *3*, Hep G2 cells; lane *4*, control for lane *3* (no template).

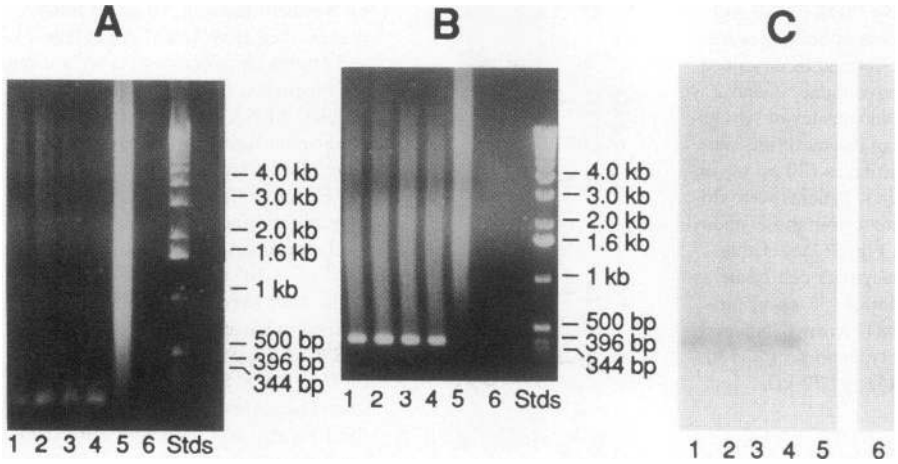

Figure 7. Qualitative demonstration of PC-1 transcription in chondrocytes and plasma cells by RNA–PCR. (*A*) RNA–PCR for housekeeping gene L30 performed from 5 μg of total RNA as described in Methods. Lane *1*, human chondrocytes, second passage; lane *2*, chondrocytes cultured in 100 μM dexamethasone; lane *3*, chondrocytes cultured with recombinant TGFβ 1 μg/ml; lane *4*, NS-1 cells; lane *5*, no template control, nonsense primers; lane *6*, no template control for lane *1*. Lanes *1–4* yielded a PCR product of the expected size for L30 (196 bp). (*B*) RNA–PCR for PC-1. Lanes *1–4* as in *A*. Lane *5*, no template control; lane *6*, no sample. Lanes *1–4* yielded a PCR product of the expected size (381 bp) for PC-1. (*C*) Southern blotting verification of hybridization of PC-1 RNA–PCR product with ^{32}P-labeled full-length PC-1 cDNA probe. Lanes *1–5* as in *B*. Lane *6* contains L30 PCR product from *A*, lane *2*, as negative control.

(Fig. 7 *A*). The specificity of PC-1 detection by RNA–PCR was confirmed by demonstrating that the 370-bp products of the PC-1 RNA–PCR reaction, but not the 160-bp products of the L30 RNA–PCR reaction, hybridized with a full-length human PC-1 cDNA probe (Fig. 7 *C*).

Therefore, PC-1 was constitutively expressed as a low abundance mRNA by cultured osteosarcoma cells and articular chondrocytes.

Regulation of PC-1 expression in osteosarcoma cells by TGFβ. The constitutively low expression of PC-1 observed in cultured cells from bone and cartilage suggested the potential importance of identifying physiologically significant factors that could upregulate PC-1 expression. Recently, Oyajobi et al. (48) demonstrated that coculture (for 72 h) with the bone and cartilage growth and repair factor TGFβ1 significantly increased NTPPPH activity in primary cultures of osteoblast-like cells from the trabecular bone of adult human femora. Thus, we first verified the functional significance of the PC-1 expressed by human osteosarcoma cells by immunoprecipitation (Table I). We verified that antibodies to PC-1, but not preimmune IgG, or staph protein A–Sepharose alone immunoprecipitated NTPPPH (5′-nucleotide phosphodiesterase I) activity from murine NS-1 plasmacytoma cell lysates. Under these conditions, anti–PC-1 precipitated enzyme activity from lysates of human osteosarcoma cells, at the same time depleting this activity from the lysates (Table I). Thus, PC-1 was expressed in osteosarcoma cells in a functionally active form.

TGFβ1 significantly increased cellular NTPPPH activity in U2OS osteosarcoma cells (Table II), in association with the detection of de novo synthesized [^{35}S]methionine-labeled PC-1 polypeptides (Fig. 8 *A*). Analysis of PC-1 gene expression in osteosarcoma cells by RNA–PCR suggested a TGFβ-induced increase in PC-1 mRNA (Fig. 8 *B*). This was confirmed by noting upregulation of PC-1 mRNA (3.8 kb) by Northern blotting (Fig. 8, *C* and *D*) where PC-1 mRNA could be detected easily in the TGFβ-stimulated cells but not control cells using only 15 μg of total RNA. Thus, TGFβ upregulated both PC-1 gene expression and 5′-nucleotide phosphodiesterase I/NTPPPH in osteosarcoma cells.

Table I. Immunoprecipitation of 5′-Nucleotide Phosphodiesterase I Activity from Murine Plasmacytoma (NS-1) and Human Osteosarcoma Cells (U2OS) by Antibodies to PC-1

Conditions	NS-1 cells OD 405	NS-1 cells	U2OS cells OD 405	U2OS cells
		enzyme U/ml		*enzyme U/ml*
Sup/no IgG	0.592±0.010	7.74±0.14	0.703±0.026	9.2±0.34
Beads/no IgG	0.060±0.002	0.78±0.02	0.064±0.006	0.84±0.08
Sup/preimmune IgG	0.601±0.028	7.85±0.36	0.701±0.016	9.2±0.21
Beads/preimmune IgG	0.062±0.003	0.82±0.04	0.064±0.001	0.84±0.03
Sup/anti–PC-1 IgG	0.555±0.005	7.26±0.06	0.435±0.065	5.7±0.84
Beads/anti–PC-1 IgG	0.173±0.008	2.27±0.11	0.358±0.043	4.7±0.55

Triton X-100 cell lysates (40 μg protein in a total volume of 0.02 ml) were incubated in triplicate with 0.01 ml of nonimmune rabbit IgG, or the same volume of anti–PC-1 IgG, and with Staph protein A–Sepharose beads (0.05 ml of a 50% suspension) as described in Methods. Sedimented, washed beads, and the remaining supernatant (*Sup*) were assayed for 5′-nucleotide phosphodiesterase I as described in Methods. Enzyme units are expressed in micromoles of substrate hydrolyzed per hour per sample. Values are representative of four separate experiments.

Table II. TGFβ Increases 5′-Nucleotide Phosphodiesterase I in U2OS Osteosarcoma Cells

Conditions	OD 405	Enzyme U/50 μg cell protein (±SD)
U2OS cells (control)	0.332±0.01	7.8±0.24
U2OS cells plus TGFβ	0.395±0.01*	9.3±0.15*

Confluent U2OS cells were incubated for 24 h (within a Falcon 3046 culture plate [Falcon Labware, Oxnard, CA] in 3 ml McCoy's 5A with 15% FCS), as described above, with either no addition (*control*) or the presence of human recombinant TGFβ1 (1 ng/ml). Cells were harvested and then Triton-lysed, and 5′-nucleotide phosphodiesterase I activity was measured in aliquots (n = 3) that contained 50 μg of cellular protein, as described above. The results indicated were from cells sampled at the same passage as in the experiment outlined in Fig. 8, *A–D* (results were similar in three other experiments). * $P < 0.01$ for TGFβ relative to control.

Discussion

NTPPPH activity is implicated in physiologic and pathologic skeletal and cartilage mineralization (21, 22). This study is the first to establish a molecular identity for an NTPPPH enzyme species in cells derived from human bone and cartilage. We hypothesized that, like certain other ectoenzyme genes (e.g., alkaline phosphatase), at least one NTPPPH might have a closely related identity in cells from liver, bone, and cartilage and in certain differentiated leukocytes. We demonstrated that expression of PC-1, an NTPPPH/5′-nucleotide phosphodiesterase I (previously detected in murine plasma cells [32, 34], human fibroblasts [47], and bovine hepatocytes [35]), is a property shared by human HepG2 hepatoma cells, chondrocytes, and osteoblast-like U2OS osteosarcoma cells (40).

We observed that human osteosarcoma cells, chondrocytes, hepatoma cells, and murine plasmacytoma cells expressed PC-1 polypeptides as species of variable size (including those of ~ 230–260, 115–130, 90, and 60 kD). We also detected PC-1 expression not only in vitro but also in vivo (from homogenized human cartilage [Fig. 3]). Full-length PC-1 is a glycosylated transmembrane homodimer of 115–130 kD (33, 34). Incomplete reduction of the intracellular interchain disulfide bond in the relatively inaccessible transmembrane region of PC-1 (39) likely accounts for detection of some of the larger species, as in Figs. 1, 2, and 8 *A*. Previous observations on COS cells transfected with PC-1, and on untransfected plasma cells, suggest that PC-1 polypeptides may also be expressed as soluble forms with lower molecular masses of 45–92 kD (37, 39). Furthermore, the primary structure of PC-1 contains numerous potential proteolytic cleavage sites (33, 34, 39). In this regard, the appearance of less than full-length cross-reactive species of PC-1 (including the 60-kD species) increases with time in storage in lysates of osteosarcoma cells and chondrocytes and in cells transfected with PC-1 cDNA (38), suggesting possible degradative events (data not shown). Thus, it will be important in further studies to define potential proteolytic processing and/or alternative splicing of PC-1 in these tissues and to define the enzymatic properties of individual processed PC-1 polypeptides.

Interestingly, various soluble forms of 5′-nucleotide phosphodiesterase I have been described in serum (49–51). Further-

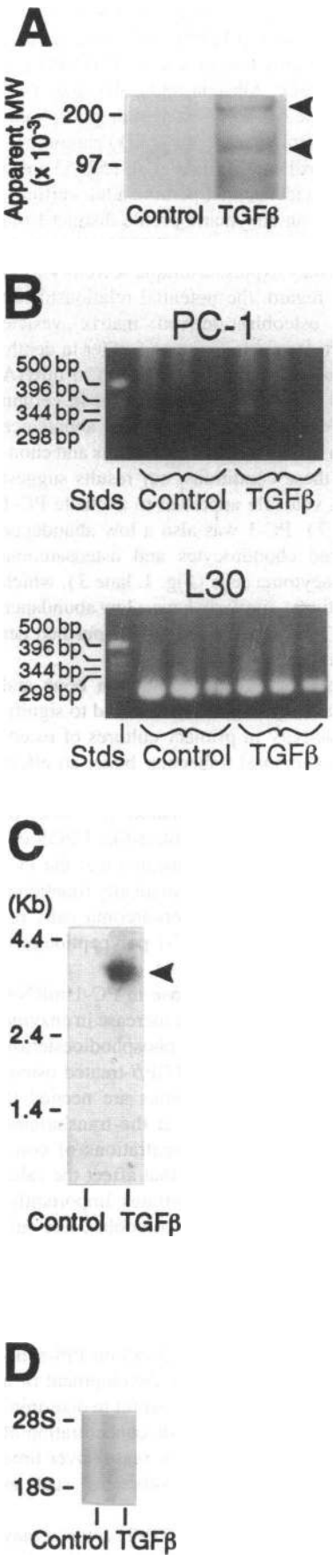

Figure 8. TGFβ upregulates PC-1 expression in osteosarcoma cells. All experiments were done using cells at the same passage as in Table II. (*A*) Human U2OS osteosarcoma cells (~ 0.5 × 10^6 cells) labeled with [^{35}S]methionine were left untreated in complete medium with 15% FCS or treated in the same medium with added human recombinant TGFβ1 (1 ng/ml) for 24 h, and cell lysates were then immunoprecipated with anti–PC-1 antibodies and analyzed by SDS/PAGE and enhanced autoradiography as described in Methods. Arrows indicate precipitated PC-1 polypeptides of ~ 130 and 260 kD in TGFβ-treated cells. Control precipitations with nonimmune sera were negative (not shown). (*B*) Total RNA (5 μg) from the untreated and TGFβ-treated osteosarcoma cells was isolated at 24 h, reverse-transcribed, and analyzed for PC-1 and housekeeping gene (L30) mRNA by nested RNA–PCR as described above. In the electrophoresis gels indicated, 20% of each final PCR product was serially diluted twice at 1:1 (proceeding left to right on gels). More PC-1 mRNA (but equivalent amounts of L30 mRNA) was detectable in TGFβ-treated cells by these methods. (*C*) Total RNA (~ 15 μg) from the untreated and TGFβ-treated osteosarcoma cells was analyzed by Northern blotting using random-primed ^{32}P-labeled human recombinant PC-1 as described above. The autoradiograph was exposed for 4 d at −70°C. RNA size standards are indicated. *Arrowhead*, 3.8 kb PC-1 mRNA. (*D*) Ethidium bromide–stained gel from *C* above as it appeared before transfer.

more, erythrocytes express a unique NTPPPH selective for inosine triphosphate (1). Thus, genes other than PC-1 may encode 5′-nucleotide phosphodiesterase I/NTPPPH in human tissues. In this regard, PC-1 may be only one of several NTPPPH enzymes in cartilage. Specifically, Masuda and colleagues (52, 53) recently purified a soluble 61-kD 5′-nucleotide phosphodiesterase I/NTPPPH derived from a larger (127 kD) parent molecule in porcine articular cartilage. Masuda et al. (52, 53) also have provided preliminary evidence that porcine adult cartilage matrix vesicle NTPPPH has an enzymatic profile distinct from that of cartilage chondrocyte membrane NTPPPH. Thus, it is possible that chondrocytes may express a unique secretory form of NTPPPH (53). In this regard, the potential relationship of PC-1 to cartilage and osteoblast-derived matrix vesicle NTPPPH will be an important area to examine further in depth.

Regulation of PC-1 expression. The amount of PC-1 mRNA is constitutively quite low in human fibroblasts and in certain other tissues (33, 47). We similarly observed low abundance PC-1 mRNA expression in resting osteosarcoma cells and chondrocytes (Fig. 5). Under these conditions, our results suggest that RNA–PCR may be a valuable approach to evaluate PC-1 transcription (Figs. 6 and 7). PC-1 was also a low abundance protein product in cultured chondrocytes and osteosarcoma cells. In this regard, plasmacytoma cells (Fig. 1, lane *3*), which have high PC-1 specific activity, similarly have a low abundance of PC-1 protein, i.e., only ~ 10 μg of PC-1 can be purified per 100 g of plasmacytoma tumors (34).

TGFβ1, a growth and repair factor present in bone and cartilage matrix (48, 54), has recently been observed to significantly increase NTPPPH activity in primary cultures of osteoblast-like cells from human femoral trabecular bone, an effect attenuated by inhibitors of transcription and of translation (48). We noted that TGFβ1 also induced upregulation of 5′-nucleotide phosphodiesterase I/NTPPPH in osteoblast-like U2OS osteosarcoma cells (Table II) and we demonstrated that the PC-1 expressed by osteosarcoma cells was enzymatically functional (Table I). We also demonstrated that osteosarcoma cells responded to TGFβ1 by clearly increasing PC-1 polypeptide production and PC-1 mRNA expression (Fig. 8).

The contrast between the marked increase in PC-1 mRNA transcripts (Fig. 8) and the relatively modest increase in enzyme activity (~ 20% increase in 5′-nucleotide phosphodioesterase I activity measured over 1 h, Table II) in TGFβ-treated osteosarcoma cells was unexpected. Future studies are needed to determine whether PC-1 also is regulated at the translational level or whether TGFβ can alter the concentrations of compounds (e.g., nucleoside monophosphates) that affect the catalytic rate of NTPPPH (6, 9) for different substrates. Importantly, the effect of TGFβ on NTPPPH in primary osteoblast-like cultures is characterized by a progressive rise (that reaches a two- to threefold increase) in extracellular PPi generation in the presence of added extracellular ATP over 72 h in culture (48). The sustained elevation of PPi concentration under these conditions may be partially mediated by an inhibitory effect on PPi-splitting alkaline phosphatase activity (48). The development of a specific immunoassay for PC-1 should prove useful in determining whether small increases in the polypeptide concentration of PC-1 in cells are sufficient to induce large increases over time in the degradation of individual NTPPPH substrates such as ATP.

Interestingly, the effects of TGFβ on NTPPPH activity may be tissue specific. Specifically, human dermal fibroblast NTPPPH activity (which appears largely attributable to PC-1 [47]) is not upregulated by TGFβ (48). In contrast, Rosenthal et al. (55) have shown that TGFβ affects a modest (~ 10%) but significant increase in plasma membrane NTPPPH in porcine articular cartilage chondrocytes. We addressed only qualitative expression of PC-1 mRNA in chondrocytes, detecting expression by RNA–PCR after TGFβ stimulation (Fig. 7). More detailed assessment of the effects of TGFβ and other growth and repair factors on PC-1 expression in fibroblasts and chondrocytes is an important area for further study.

NTPPPH/PC-1 is expressed as an ectoenzyme on not only the plasma membrane but also in the rough endoplasmic reticulum (31, 37). Importantly, upregulation of PC-1 expression by cDNA transfection markedly increases not only extracellular PPi generation but also intracellular PPi generation (38). Furthermore, the latter effect does not require supplementation with exogenous ATP (38). Thus, it will be important in future studies to test the hypothesis that transcriptional upregulation of PC-1 by TGFβ and other bone and cartilage growth factors may be one mechanism by which cells specialized for mineralization modulate rapid increases in PPi generation.

In conclusion, the identification of PC-1 as an NTPPPH expressed by cells derived from cartilage and bone may provide a novel molecular tool to further understand the role of NTPPPH expression in both physiologic and pathologic bone and cartilage mineralization.

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